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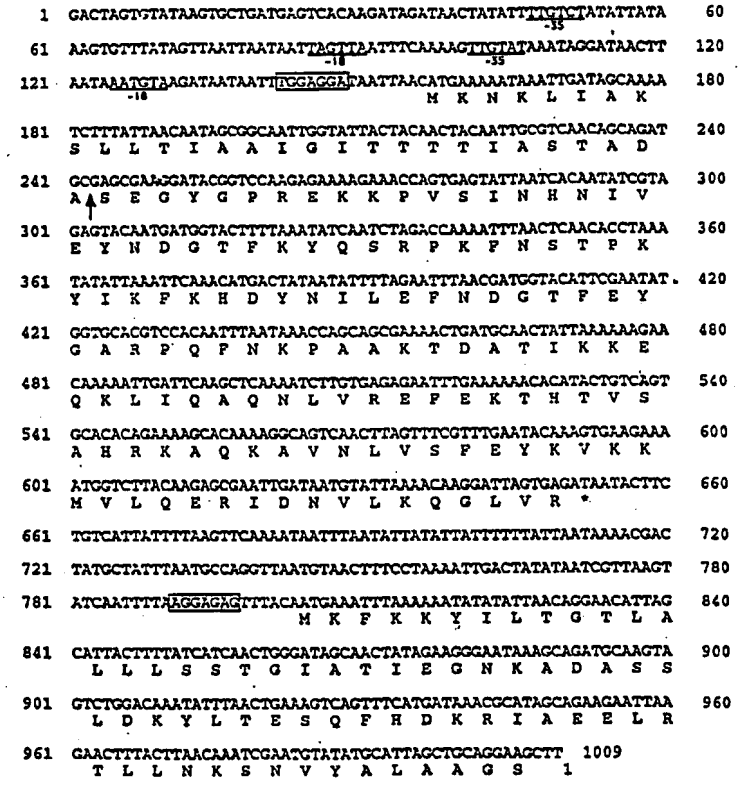
(54) Title: FIBRINOGEN BINDING PROTEIN

(57) Abstract

The present invention relates to new fibrinogen binding proteins derived from Staphylococci, having a molecular weight of 19 kDa and/or 60 kDa, respectively.



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FIBRINOGEN BINDING PROTEIN

DESCRIPTION

Technical field

The present invention relates to fibrinogen binding proteins. Further the invention relates to pharmaceutical compositions and method for treatment.

The object of the present invention is to obtain fibrinogen binding proteins.

A further object is to obtain said protein by means of genetic engineering technique by using, e.g. a plasmid comprising a nucleotide sequence coding for said protein.

Background of the invention

Clumping of Staphylococcus aureus in plasma has been suggested as a potential virulence factor.[1-5] Several mechanisms can be responsible for this aggregation. A fibronectin-binding protein has been suggested to cause aggregation of staphylococci in fibronectin at the concentration found in sera.[5,6] The presence of protein A causes staphylococci to aggregate in normal human sera, which frequently contain specific immunoglobulins directed against staphylococcal antigens.[7] Due to a high cell surface hydrophobicity, many staphylococcal strains autoregulate under isotonic conditions.[8] It is believed that clumping of staphylococci in fibrinogen is caused by the so called clumping factor or fibrinogen-binding protein, situated on the staphylococcal cell surface.[1,9] Fibrinogen has also been suggested to mediate adhesion of S. aureus to cultured human endothelial cells[10] and to catheters in vitro and in vivo.[11,12] It has been disputed whether clumping factor is distinct from coagulase[1] or if it is a cell-bound form of coagulase.[13,14] Staphylococcus aureus coagulases can be grouped into eight different serotypes[15] and the existence of multiple molecular forms of coagulases

has been suggested,[16] although most investigators believe that lower molecular weight subspecies in coagulase preparations are due to proteolytic degradation of a larger protein.[17] Staphylococcal coagulases have been shown to induce polymerization of fibrinogen to fibrin by binding, and thereby activating, prothrombin. The coagulase-prothrombin complex causes the release of fibrinopeptides from fibrinogen in a manner similar to that described for thrombin in physiological blood clotting.[18] Fibrinogen precipitation and network formation can also be induced non-enzymatically, e.g. by exposing fibrinogen to various highly positively charged molecules like protamine, which interacts with specific negatively charged sites on the D-domain of fibrinogen.[19]

We have recently described staphylococcal components that interact with fibrinogen and which can be purified from S. aureus culture supernatants.[13] These are an 87 kDa coagulase and a 19 kDa fibrinogen-binding protein. The 87 and 19 kDa fibrinogen-binding proteins are essentially extracellular proteins, but can to some extent be found on the staphylococcal cell surface. Thus, these proteins can give rise to the clumping phenomenon both by inducing coagulation and by direct fibrinogen-binding.

In this report we show that there are at least three distinct fibrinogen-binding proteins produced by S. aureus strain Newman, and that two of these proteins are coagulases.

Results

SDS-PAGE analysis of fibrinogen binding-proteins produced at different times during staphylococcal cell growth

Staphylococcus aureus strain Newman was grown in BHI or LB and samples were taken every hour for 14 h. Culture supernatants were applied onto fibrinogen-Sepharose

and the eluted material was analysed on Coomassie blue-stained SDS-PAGE gels. Figure 1 shows fibrinogen-binding proteins from culture supernatants of staphylococci grown in LB under low aeration conditions. Under these conditions, an 87 kDa protein was produced in large amounts, mainly during the first 7 h and a 60 kDa protein appeared after 5-6 h and was produced in large amounts after 9 h of growth. Under high aeration conditions, the 87 kDa protein was produced in lower amounts and the switch to production of the 60 kDa protein accurred after only 3 h resulting in a higher production of 60 kDa protein compared to when less air was supplied to the culture. Using a rich medium like BHI, and the same high aeration conditions, this switch again accurred after 7 h (data not shown). In all cultures, the 87 kDa protein was produced mainly during the exponential growth phase and the 60 kDa protein mainly during the post-exponential growth phase. The switch from production of the 87 kDa protein to production of the 60 kDa protein reflected the nutritional status, rather than the optical density of the culture. A 19 kDa protein was produced constitutively during these 14 h of growth (Fig. 1).

SDS-PAGE, affinity- and immuno-blot analysis of affinity purified proteins

Staphylococcus aureus grown in BHI for 3-4 h produced the 87 and 19 kDa proteins but no detectable 60 kDa protein. Such culture supernatants were applied onto fibrinogen-Sepharose in order to purify the 87 and 19 kDa proteins. Similarly, culture supernatants from S. aureus grown in LB for 6-8 h, containing predominantly the 60 kDa protein but also the 87 and 19 kDa proteins, were used to purify the 60 kDa protein. The crude material was first passed over fibrinogen-Sepharose, in order to eliminate the 87 and 19 kDa proteins, and the effluent (containing the 60 kDa protein which also bound to fibrinogen-Sepharose, but

to a lower extent than the 87 and 19 kDa proteins) was applied onto prothrombin-Sepharose. The 87 and 19 kDa proteins did not bind to prothrombin-Sepharose. Eluted material from affinity purifications was subjected to SDS-PAGE and affinity-blot analysis (Fig. 2). These blots were probed with fibrinogen or prothrombin, followed by rabbit antifibrinogen or rabbit antiprothrombin sera which had been pre-incubated with S. aureus culture supernatants in order to absorb naturally occuring antistaphylococcal antibodies. It could thus be shown that the 87 and 19 kDa proteins bound only to fibrinogen and not to prothrombin, while the 60 kDa protein bound both fibrinogen and prothrombin. Controls were performed by incubating filters with only pre-absorbed primary antibody, omitting fibrinogen and prothrombin (data not shown). In these controls, no 87, 60 or 19 kDa proteins were detected. By using a dilution series both of antigen and fibrinogen or prothrombin, it was shown that the binding reactions were specific and not the result if contaminating blood proteins in the fibrinogen and prothrombin preparations. For example, 10 ng/ml of fibrinogen could detect 0.1 ng of the 87 or 60 kDa proteins in these affinity-blots. When 10 ng/ml of prothrombin was used in these tests, 0.1 ng 60-kDa protein could be detected, while a concentration of 10 µg/ml of prothrombin could not detect a 1 ng 87-kDa band (data not shown).

The anti-19 serum recognized not only the 19 kDa protein but also the 87 kDa protein and a 35 kDa protein (Fig. 3). Furthermore, there was a close resemblance between blots incubated with fibrinogen followed by anti-fibrinogen antibody and blots incubated with anti-19 serum.

Antibodies to the 60 kDa protein seem to occur naturally among several mammalian species (e.g. rabbit, goat and man; data not shown). The anti-19 serum, as well as pre-immune serum from the same rabbit, showed some re-

activity towards this 60 kDa protein. However, pre-absorption with 19 kDa protein completely abolished binding to the 19 and 35 kDa bands, but not to the 60 kDa band, while antiserum pre-absorbed with 60 kDa protein reacted with the 19 and 35 kDa bands but not with the 60 kDa band (Fig. 4).

Peptide mapping

Proteins were purified by a combination of affinity chromatography and preparative SDS-PAGE. The purity of these preparations was confirmed on silver stained SDS-PAGE gels (Fig. 5). Dimerisation of the 19 kDa protein into a 35 kDa protein could be detected on the silver stained gels. On affinity-blots, using fibrinogen and antifibrinogen antibodies, not only the 35 kDa dimer, but also bands of higher molecular weight were detected. Upon digestion with α-chymotrypsin, the dimerisation of the 19 kDa protein was disrupted, but the 19 kDa band was left intact. This protease did not have any apparent effect on the 87 kDa protein, whereas the fibrinogen-binding ability of the 60 kDa protein was completely lost after treatment with α-chymotrypsin. On the contrary, treatment of these proteins with staphylococcal V8 protease only partly digested the 60 kDa protein while the 87-kDa protein was digested into low molecular weight peptides (Fig. 5).

NH_2-terminal sequence analysis

Analyses of NH_2-terminal sequences revealed that the 87 kDa protein was related to previously described coagulases, while the 19 kDa protein had a unique NH_2-terminal sequence. The NH_2-terminal sequence of the 60 kDa coagulase was blocked (Table 1).

Coagulase test

Coagulase tests were performed with proteins purified by a combination of affinity chromatography and

preparative SDS-PAGE. These preparations did not contain contaminations of other staphylococcal proteins as shown on silver stained SDS-PAGE gels (Fig. 5). The 87 and 60 kDa proteins coagulated rabbit plasma, while the 19 and 35 kDa proteins produced a precipitate or a weak coagulase reaction in these

Table 1 NH_2-terminal sequence analysis

Staphylococcal strain	NH_2-terminal sequence
S. aureus BB	IVTKD YSKES RVNEN SKYGT
S. aureus 213	IVTKD YSKES RVNEK SKKGA
S. aureus 8325-4	IVTKD YSGKS QVNAG SKNGT
S. aureus Newman 87 kDa	IVTKD YSGKS QVNAG SKNGT
S. aureus Newman 60 kDa	-
S. aureus Newman 19 kDa	SEGYG PREKK PVSIN HNIVE
S. aureus Newman 35 kDa	M-Y- P-EKK PV-

Table 2 Coagulase test

Preparation	Inhibitor	Clotting at 1 h	2 h	24 h
87 kDa	-	+	+	+
87 kDa	Aprotinin,PMSF,NEM,EDTA	+	+	+
87 kDa	Heparin	-	+	+
87 kDa	DFP	-	-	-
60 kDa	-	+	+	+
60 kDa	Aprotinin,PMSF,NEM,EDTA	+	+	+
60 kDa	Heparin	-	+	+
60 kDa	DFP	-	-	-

tests. Dilution series of the 87 and 60 kDa proteins showed that there was a dose-response relationship. To produce a positive reaction, 25 ng of the 60 kDa protein was re-

quired, while only 1 ng of the 87 kDa protein was needed to coagulate rabbit plasma within 24 h. The activities of the 87 and 60 kDa coagulases were not affected by the addition of the protease inhibitors NEM, EDTA, aprotinin and PMSF, to the rabbit plasma. In the presence of heparin, higher concentrations of both coagulases were needed for a positive reaction. Rabbit plasma containing the protease inhibitor DFP was not clotted by either coagulase (Table 2).

Identification of the 19 kDa fibrinogen-binding (fib) protein from strain FDA 486.

S. aureus strain FDA 486, which was the strain from which the library was obtained, was shown to express the 19 kDa fib protein. Affinity purified material from staphylococcal culture supernatants were analyzed in Western blots after SDS-PAGE separation (Fig. 1). The 19 kDa fibrinogen-binding protein expressed by the FDA 486 strain bound fibrinogen and the anti-fib serum, comparable to the protein purified from strain Newman.

Cloning of the fib gene in E. coli.

A genomic library containing DNA from S. aureus strain FDA 486 was screened with the anti-fib serum. A clone designated λfib-50 was isolated. This clone expressed a fibrinogen-binding protein of approximately 16 kDa, which bound anti-fib serum in a Western blot experiment. The fib gene was further subcloned into a pBluescript SK+vector (Fig. 2). Digestion of λfib-50 with HindIII generated 3 fragments containing staphylococcal DNA. One of these, contained by pBfibIII expressed the fibrinogen-binding protein (Fig. 1). The 2.4 kb insert in the pBfibIII plasmid was isolated and digested with XbaI, resulting in two fragments of 1.7 and 0.7 kb respectively. These fragments were subcloned into the pBluescript SK+ vector as well as the M13mp18 and M13mp19 vectors (Fig. 2). Of the resulting

plasmids the pBfibT was found to express a fibrinogen-binding protein which was slightly larger than the recombinant protein produced by the pBfibIII plasmid (Fig. 1).

The invention further comprises a microorganism containing at least one hybrid-DNA-molecule according to above. The plasmid pBfibIII in an E. coli XL has been deposited at the Deutsche Sammlung von Mikroorganismen (DSM), and has thereby obtained deposition number DSM .....

Sequencing of the fib gene from S. aureus FDA 486.

The fib gene contained in the M13 constructs was sequenced by the Sanger dideoxy-chain termination method. The pBfibT plasmid was found to contain an open reading frame of 309 bp. The pBfibJ vector contained a putative TGGAGGA ribosomal binding site situated 15-9 base pairs upstream from the ATG start codon. Furhter upstream putative promoter sequences were identified. Computer assisted analysis revealed an open reading frame of 495 bp corresponding to 165 amino acids including a signal sequence of 29 amino acids. The first 23 amino acids in the mature protein were identical to the sequence obtained by NH_2-terminal analysis of the purified native protein. The predicted molecular mass of the fib protein is 15.9 kb. The complete nucleotide and deduced amino acid sequence is shown in Fig. 3.

Sequencing of the fib gene from S. aureus Newman.

Based on the DNA sequence obtained from the fib gene cloned from S. aureus FDA 486, primers were produced (Fig. 2). These primers were used to ampify the fib gene, both from strain FDA 486 and from strain Newman, using the polymerase chain reaction method. The resulting fragments were sequenced using fluorescent base terminators on an automatic sequenator. A comparison between the fib genes from these staphylococcal strains is shown in Fig. 4. The

sequence from the cloned fib gene from S. aureus FDA 486 was confirmed using the same sequencing strategy. A comparison between the deduced amino acid sequences of the fib proteins from the two strains is shown in Fig. 5.

Computer assisted analysis of the fib gene sequence.

Using either the nucleotide or the amino acid sequence as a probe, no close similarity to any protein or nucleotide sequence in the University of Wisconsin Genetics Computer Group database was found. The protein showing the closest resemblance was coagulase from S. aureus. A sequence of 22 amino acids repeated twice with a spacing of 9 amino acids, located in the NH_2-terminal part of the mature protein showed homology to the COOH-terminal part of coagulase, where several 27 amino acids long repeats are situated (Fig. 6).

Discussion

We have previously described a 87 kDa fibrinogen-binding protein which exerts coagulase activity and is produced by S. aureus in culture supernatants.[13] We have suggested that this 87 kDa coagulase and a 19 kDa fibrinogen-binding protein, both of which are present on the cell surface, are involved in the clumping of S. aureus in fibrinogen. In this study we show that S. aureus strain Newman has two different types of coagulase secreted in a sequential manner during cell growth (Fig. 1). The 87 kDa coagulase was produced early during growth and was later replaced by the 60 kDa coagulase. The rate at which this switch occurred varied with growth rate and type of media used, i.e. under low aeration conditions or in a rich medium this switch was postponed (data not shown). This suggests that the presence of some environmental factor(s) induces the production of the 87 kDa protein and suppresses 60 kDa protein production. It is likely that the 87 kDa

coagulase is negatively regulated by the agr locus together with protein A.[20]

It was concluded from the results of the analyses by SDS-PAGE and immunoblotting of proteins purified by affinity chromatography that both the 60 and 87 kDa proteins bound fibrinogen, but only the 60 kDa protein bound prothrombin (Fig. 2). This is contradictory to our previous results where the 87 kDa protein was shown to bind prothrombin.[13] In these earlier experiments, 10 µg/ml prothrombin was used. This was unfortunate, as we have since shown that contamination with 1 ng/ml fibrinogen can detect a band of 100 ng of fibrinogen-binding protein in immunoblot experiments. When antigens were diluted to 1 or 0.1 ng per band and ligands were used at 10 ng/ml, background due to contamination in these preparations was eliminated (data not shown).

Thus the following nucleotide sequence is present in the gene coding for said protein:

GAGCGAAGGA TACGGTCCAA GAGAAAAGAA ACCAGTGAGT ATTAATCACA
ATATCGTAGA GTACAATGAT GGTACTTTTA AATATCAATC TAGACCAAAA
TTTAACTCAA CACCTAAATA TATTAAATTC AAACATGACT ATAATATTTT
AGAATTTAAC GATGGTACAT TCGAATATGG TGCACGTCCA CAATTTAATA
AACCAGCAGC GAAAACTGAT GCAACTATTA AAAAAGAACA AAAATTGATT
CAAGCTCAAA ATCTTGTGAG AGAATTTGAA AAAACACATA CTGTCAGTGC
ACACAGAAAA GCACAAAAGG CAGTCAACTT AGTTTCGTTT GAATACAAAG
TGAAGAAAAT GGTCTTACAA GAGCGAATTG ATAATGTATT AAAACAAGGA
TTAGTGAG

whereby this nucleotide sequence encodes for the following protein starting at nucleotide 243: (In Fig. 3 nucleotides 156-242 encode a signal peptide.)

SEGYGPREKK PVSINHNIVE YNDGTFKYQS RPKFNSTPKY IKFKHDYNIL
EFNDGTFEYG ARPQFNKPAA KTDATIKKEQ KLIQAQNLVR EFEKTHTVSA
HRKAQKAVNL VSFEYKVKKM VLQERIDNVL KQGLVR

Although antisera to the 19 kDa protein recognized the 87 kDa protein (Fig. 3), pre-absorbtion with 19 kDa protein, which could eliminate the binding to the 19 kDa protein, could not completely abolish this binding to the 87 kDa protein. In addition, antisera to the 87 kDa protein did not specifically recognize the 19 kDa protein (data not shown). The immunological cross-reactivity could be due to structural similarities in the fibrinogen-binding sites of these proteins. Antisera to the 19 kDa protein also recognized the 35 kDa protein (Fig. 3). We have previously shown that the 19 kDa protein spontaneously forms 35 kDa dimers (not reducible with 2-mercapto ethanol) and to a lesser extent higher molecular weight bands that seem to be trimers and tetramers of this protein.[13] Minor bands in the preparation could thus be due to further aggregation of the 19 kDa protein or to degradation of the 87 kDa protein (Fig. 3). By pre-absorbing the rabbit anti-19 serum with either 19 or 60 kDa proteins, it was shown that there were no shared antigenic epitopes between the 60 kDa protein and 19 kDa protein (Fig. 4). It is likely that antibodies against the 60 kDa protein are present in the most normal rabbit sera. This reactivity is not due to unspecific binding to immunoglobulins. The purified 60 kDa protein did not bind control antibodies in immunoblots, and was thus shown not to contain protein A activity.

Peptide mapping analysis suggested that the 87, 60 and 19 kDa proteins are not closely related (Fig. 5). It was shown that digestion with α-chymotrypsin and staphylococcal V8 protease gave different peptide banding patterns with the three different proteins, and that the 60 kDa protein completely lost its ability to bind fibrinogen upon digestion with α-chymotrypsin, whereas the 87 and 19 kDa proteins were unaffected.

Analyses of NH_2-terminal sequences suggested that the 87 kDa coagulase is identical to the coagulase from

strain 8325-4 (Table 1). This is in agreement with the fact that these strains produce coagulases of the same serotype. The NH_2-terminal sequence of the 19 kDa protein was not homologous to any of the previously described coagulases. Computer-assisted analysis revealed that this NH_2-terminal sequence is a new, unique sequence.[21] The strong similarity between this sequence and the NH_2-terminal sequence of the 35 kDa protein further strengthens the evidence that the 35 kDa protein is a dimer of the 19 kDa protein. The 60 kDa coagulase seems to represent a third type of fibrinogen-binding protein from S. aureus.

In the coagulase test, it was shown that highly purified preparations of both the 87 and 60 kDa proteins exerted coagulase activity (Table 2). There was a clear dose-response relationship when dilution series of the 87 and 60 kDa proteins were subjected to coagulase tests (data not shown). It appears that the 87 kDa coagulase had a higher specific activity than the 60 kDa coagulase; however, the treatment of these proteins during purification could have influenced their coagulase activities (for example the 60 kDa coagulase seemed to be more sensitive than the 87 kDa coagulase to exposure to the acetic acid used in the elution step). These clotting reactions were due to true coagulase reactivity, since no inhibition could be achieved by addition of protease inhibitors.[22,23] Addition of heparin influenced the clotting of both coagulases, such that a higher concentration of coagulase was needed for positive tests. Since heparin inhibits physiological blood clotting, we suggest that this negative effect is not due to direct interaction of heparin with staphylococcal coagulases. DFP, which is a powerful protease inhibitor, has previously been shown to inhibit both thrombin- and coagulase-mediated plasma clotting.[24] Addition of DFP to rabbit plasma inhibited clotting by both coagulases.

The amino acid sequence of a 43 kDa fragment from the S. aureus 213 (serotype II) coagulase has been determined.[25] The coagulase activity is contained in the NH_2-terminal region of this molecule, and the prothrombin-binding capacity resides further downstream within this region.[26] Coagulases from S. aureus BB (serotype I), S. aureus 213 and S. aureus 8325-4 (serotype III) have recently been expressed in E. coli.[27-29] The amino acid sequences of these recombinant coagulases reveal a quite strong homology (>90%) in the C-terminal halves of the molecules, while the NH_2-terminal regions show only approximately 50% homology.[30] The gene clones encoding these coagulases do not contain sequences that correspond to the NH_2-terminal sequence of the 19 kDa protein. The 87 kDa coagulase from strain Newman (serotype III) seems to be identical to the coagulase from S. aureus 8325-4. Coagulases of serotypes I and II have been shown to bind human prothrombin.[17,18] However, it has not been established if the coagulase from strain 8325-4 binds prothrombin. It is interesting, however, that a coagulase from strain Newman, a strain of human origin, does not bind human prothrombin, although our preliminary results indicate that prothrombin is required for its function.[13] It is possible that the 60 kDa coagulase, which has a strong affinity for prothrombin and a somewhat weaker affinity for fibrinogen (as compared to the 87 kDa coagulase), has a different mode of action than the 87 kDa coagulase. The ability of the 19 kDa fibrinogen-binding protein to oligomerize, to precipitate fibrinogen in plasma and to some extent coagulate plasma, suggests that this protein could affect fibrinogen in the same manner as paracoagulating substances have been suggested to work.[19,31]

In conclusion, S. aureus strain Newman produces two distinct fibrinogen-binding coagulases. These are produced in a sequential manner during growth and have dif-

ferent binding and antigenic properties. A third fibrinogen-binding protein is a 19 kDa protein, which spontaneously forms dimers and larger aggregates. The role of coagulases and clumping factors (or fibrinogen-binding proteins as we suggest is the correct designation for these proteins) in staphylococcal virulence and pathogenicity has not yet been established. However, in our preliminary study, 90% of 40 S. aureus isolates from wound infections had coagulase activity, and among these >60% produced the 87 kDa protein. It is notable that coagulases are produced in large amounts by S. aureus and in such a fashion that there is always one type of coagulase present in the culture medium. The fact that these proteins interact specifically with host proteins makes coagulases interesting subjects for further study. An E. coli clone expressing the 19 kDa protein has been isolated and sequence determination is in progress in our laboratory.

Binding of staphylococci to fibrinogen on coated coverslips or on catheters has been described (Cheung and Fischetti, 1990; Cheung et al., 1991; Herrmann et al., 1988; Kuusela et al., 1985; Mohammad et al., 1988; Vaudaux et al., 1989). It is also a well known fact that most Staphylococcus aureus clump in the presence of fibrinogen. It has been suggested that this clumping reaction involves a small peptide on the COOH-terminal part of the gamma chain on the fibrinogen molecule (Strong et al., 1982). On the other hand, the fibrinogen-binding component of the staphylococci remained elusive for a long time. It was suggested that a fibrinogen-binding protein would be attached to the staphylococcal cell surface (Duthie, 1954; Espersen, 1987; Jeljaszewicz et al., 1983). We have identified 3 different fibrinogen-binding proteins from Staphylococcus aureus, all of which can be found on the staphylococcal cell surface (Bodén and Flock, 1992). However, these proteins cannot be described as cell surface proteins

because they are mainly expressed extracellularly. In addition one of the identified fibrinogen-binding proteins was found to be coagulase, a well known extracellular staphylococcal protein. The other fibrinogen-binding proteins were a second type of coagulase of 60 kDa and a 19 kDa fibrinogen-binding protein without coagulase activity. In this report we describe the cloning, expression and sequencing of this 19 kDa protein.

Expression of recombinant protein from the λ cloned isolated and the subclones of this was investigated (Fig. 1). The λ clone and the pBfibIII subclone both expressed a 15 kDa protein which bound fibrinogen and the anti-fib serum, whereas strain pBfibT expressed a protein of approximately 18 kDa. This was due to the fact that this construct contained a fusion product between the β-gal protein and the fib protein, lacking the first 33 amino acids. All recombinant proteins seemed to bind fibrinogen to a lesser extent than the native protein from S. aureus, since the binding of fibrinogen gave a weaker response than the binding of anti-fib serum in immunoblots. The opposite was true for the native protein.

The nucleotide sequence of the intact fib gene revealed an open reading frame of 165 amino acids, including a signal sequence of 29 amino acids (Fig. 3). The signal sequence had the characteristics of a typical signal sequence (Pugsley, 1989), such as a net positive charged region spanning the first 8 residues at the NH_2-terminus, a central core of 5 hydrophobic and 9 neutral residues with a strong probability of forming an α-helix, a turn-inducing alanine 6 residues downstream from the cleavage site, alanines at position -3 and -1 before the cleavage site and glutamic acid at position +2 in the mature protein, creating a net positive charge difference between the NH_2-terminus of the signal peptide and the NH_2-terminus of the mature polypeptide. The first 23 amino acids of the mature

protein were completely homologous to the sequence obtained by NH_2-terminal sequence analysis of purified fib protein (Bodén and Flock, 1992). In addition, putative promote sequences and ribosomal binding site could be identified. However, there were no obvious transcription termination sequences and instead a second ribosomal binding site was identified 150 nucleotides downstream of the fib gene. This second Shine-Dalgarno sequence was followed by a start codon and a signal sequence which was homologous to the signal peptide of the fib gene. This putative peptide was not homologous to any previously described protein. One could speculate however, that these proteins are coded for by the same polycistronic transcript and thus regulated in the same way. According to our previous findings the fib protein seems to be constitutively expressed in S. aureus Newman (Bodén and Flock, 1992).

Since the previously studied protein was purified from S. aureus strain Newman the fib gene from this strain was also sequenced. Comparison between the two sequences show a remarkable resemblance (Fig. 5). The only differences being two amino acids in the signal sequence and the conservative change of the basic amino acid arginine for a likewise basic lysine at the very COOH-terminal end. On the nucleotide level there is one additional conservative nucleotide change apart from the three changes on amino acid level. Downstreams of the structural gene there are major differences between the two strains in a stretch of approximately 80 nucleotides. Closer to the putative second peptide in this transcript further downstreams the similarity is again higher, indicating that there is a similarity between the two strains also regarding this second peptide.

Computer assisted analysis of the deduced amino acid sequence suggested that there was a high probability for formation of an α-helix in the signal sequence as well

as in the last 60 to 70 residues of the COOH-terminus (). In addition the sequence NST at position 64-66, indicates that there is a glycosylation site. A glycosylation of the native protein could account for the discrepancy between the size of the native and the recombinant protein. When comparing the amino acid sequence of the fib protein to other known sequences in the database of the University of Wisconsin Genetics Computer Group, the protein with the highest similarity is coagulase from S. aureus. Coagulase from three different serotypes has been cloned and sequenced (Bodén and Flock, 1992; (Kaida et al., 1989; (Kaida et al., 1987; (Phonimdaeng et al., 1990). All of these contain repeated sequences of 27 residues in the COOH-terminus. The coagulase from strain Newman has been cloned (), but the sequence has not yet been published. A construct containing the 177 most NH_2-terminal amino acids of the Newman coagulase was shown to bind fibrinogen (McDevitt et al., 1992). In the fib protein a sequence of 22 amino acids, repeated twice with a spacing of 9 amino acids, located in the NH_2-terminal part of the mature protein showed homology to the COOH-terminal part of coagulase (Fig. 6). We suggest that this region is responsible for the fibrinogen binding.

Materials and methods

Bacterial strains and culture conditions.

Staphylococcus aureus Newman was kindly provided by M. Lindberg, Swedish University of Agricultural Sciences, Uppsala, Sweden. Staphylococci were grown overnight in Brain Heart Infusion (BHI) medium (Difco Laboratories, Detroit, MI) or in Luria-Bertani (LB) medium. After centrifugation, the bacterial pellet was resuspended in 20 culture volumes of freshly prepared BHI or LB and grown at 37°C with

constant shaking in Ehrlenmeyer flasks (low aeration) or in indented flasks (high aeration).

Affinity chromatography. Staphylococcal proteins were affinity purified as described previously.[10] Briefly, fibrinogen-Sepharose and prothrombin-Sepharose were prepared by coupling human fibrinogen (IMCO, Stockholm, Sweden) or human prothrombin (Sigma Chemical Co, St. Louis, MO) to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), by the procedure recommended by the manufacturer. The Sepharose was equilibrated with phosphate-buffered saline (PBS;145 mM NaCl, 10 mM phosphate, pH 7.4) containing 0.05% Nonidet P-40. Staphylococcal culture supernatants supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.05% Nonidet P-40 were applied. The absorbed material was eluted with 0.7% acetic acid containing 0.05% Nonidet P-40. The eluted material (eluate) was concentrated in Centricon microconcentrators (Amicon, Danvers, MA) or by acetone precipitation.

SDS-PAGE, affinity- and immuno-blotting. SDS-PAGE and subsequent diffusion blotting was performed using the PhastSystem (Pharmacia) as described previously.[10] Nitrocellulose filters were incubated for 1 h at room temperature with human fibrinogen or human prothrombin at concentrations between 1 ng/ml and 10 µg/ml in PBS supplemented with 0.05% Tween 20. Primary antibodies [rabbit anti (human)fibrinogen (Dakopatts, Glostrup, Denmark), rabbit anti(human)prothrombin (Dakopatts), and rabbit anti-19 kDa protein] were diluted 1:1000 and incubated with the filters for 2 h. The rabbit anti-19 kDa protein antibodies (anti-19 serum) were obtained by subcutaneous immunization of rabbits with a highly purified 19 kDa protein preparation emulsified in complete Freund's adjuvant. In order to eliminate naturally occurring antistaphylococcal antibodies

in rabbit antifibrinogen or rabbit antiprothrombin antisera, these were pre-absorbed with staphylococcal culture supernatants from cells grown in LB for 6 h. Undiluted antisera was added to 10 volumes of culture supernatant and incubated at room temperature for 1 h or at 4°C for 4 h before diluting the antibody to the appropriate concentration. The anti-19 serum was absorbed with 19 or 60 kDa proteins purified from preparative gels. The gel slices were homogenised in PBS containing 0.1% Nonidet P-40 before being added in a 10-fold excess to the antisera and incubated as described above. Alkaline phosphatase (ALP) conjugated goat anti-rabbit immunoglobulin G antibodies (Sigma) were diluted 1:1000 and incubated with the filters for 1 h. The ALP reaction was developed in 100 mM Tris hydrochloride (pH 8.0) containing 10 mM $MgCl_2$, 0.02 mg α-naphtylphosphate per ml (E. Merck AG, Darmstadt, Germany) and 0.02 mg Fast Blue (Merck) per ml for 10-20 min.

Purification of proteins. The 87, 60 and 19 kDa protein were purified from preparative SDS-PAGE gels by eluting proteins from gel slices in a Model 422 Electro-Eluter (Bio-Rad, Hercules, CA).

Fragmentation of proteins by proteases. Proteins were digested with 40 µg/ml of α-chymotrypsin or staphylococcal V8 protease (Sigma) for 1 h on ice.

Coagulase test. Coagulase tests were performed in Difco Coagulase Plasma (Difco) with or without the presence of the protease inhibitors N-ethylmaleimide (NEM; 2 mM), EDTA (6 mM), aprotinin (Sigma; 200 U/ml) (14), heparin (Sigma; 40 U/ml) (15), and PMSF (1 mM). Coagulate tests were also performed in the presence of 5 mM diisopropyl fluorophosphate (DFP; Janssen Chimica, Beerse, Belgium) (16).

Determination of NH_2-terminal sequences. Samples were analysed in a 470 Protein Sequencer (Applied Biosystems, Foster City, CA).

Bacterial strains and cloning vectors.

Escherichia coli strain Y1090 (Clontech, Palo Alto, CA) was used to for the screening of the λgt-11 library (Clontech) containing genomic DNA from Staphylococcus aureus strain FDA 486. For subcloning E. coli strains XL-1 and JM103 were used with the cloning vectors Bluescript SK+ (Stratagene, La Jolla, CA), pGEM7Zf(+) (Promega) and M13mp18 or M13mp19 (Promega).

Media and chemicals.

E. coli were grown in Luria Bertani medium at 37°C. Ampicillin (50 µg/ml) and tetracyclin (5 mg/ml) were added when appropriate. Restriction enzymes were purchased from Promega. IPTG and X-gal were from Boehringer-Mannheim. All other chemicals were purchased from Sigma (Sigma Chemical Co, St. Louis, MO) or Merck (E. Merck AG, Darmstadt, Germany).

Affinity chromatography

Staphylococcal proteins were affinity purified as described previously (Bodén and Flock, 1989). Briefly, fibrinogen-Sepharose was prepared by coupling human fibrinogen (IMCO, Stockholm, Sweden) to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), by the procedure recommended by the manufacturer. The Sepharose was equilibrated with phosphate-buffered saline (PBS; 145 mM NaCl, 10 mM phosphate, pH 7.4) containing 0.05% Nonidet P-40. Staphylococcal culture supernatants supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.05% Nonidet P-40 were applied. The absorbed material was eluted

with 0.7% acetic acid containing 0.05% Nonidet P-40. The eluted material was concentrated by acetone precipitation.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), affinity- and immuno-blotting.

SDS-PAGE and subsequent diffusion blotting was performed using the Phast System (Pharmacia) as described previously (Bodén and Flock, 1989). Nitrocellulose filters were incubated for 1 hour at room temperature with human fibrinogen at 10 µg/ml in PBS supplemented with 0.05% Tween 20. Primary antibodies (rabbit anti(human)fibrinogen [Dakopatts, Glostrup, Denmark] and rabbit anti-fib protein) were diluted 1:500 or 1:1000 and incubated with the filters for 2 hours. The rabbit anti-fib protein antibodies (anti-fib serum) were obtained by subcutaneous immunization of rabbits with a highly purified 19-kDa protein preparation emulsified in complete Freund's adjuvant. Alkaline phosphatase (ALP) conjugated goat anti-rabbit immunoglobulin G antibodies (Sigma) were diluted 1:1000 and incubated with the filters for 1 hour. The ALP reaction was developed in 100 mM Tris hydrochloride (pH 8.0) containing 10 mM $MgCl_2$, 0.02 mg α-naphtylphosphate per ml (E. Merck AG, Darmstadt, Germany) and 0.02 mg Fast Blue (Merck) per ml for 10-20 min.

Screening of the λgt-11 library.

Plates were grown and induction with IPTG was performed according to the protocol recommended by the manufacturer (Clontech). Nitrocellulose filters (Schleicher and Schüll) were incubated with anti-fib sera diluted 1:500, as described above.

DNA sequencing and sequence analysis.

The DNA sequence was determined by the dideoxy-chain termination method (Sanger et al 1977), using

[α-^{35}S]dATP (Amersham Corp.) and Sequenase 2.0 (United States Biochemical Corporation (USB), Cleveland, OH). Recombinant M13mp18 or M13mp19 phage was used as template. M13 Universal primer (USB) as well as custom made primers from the Unit for. Nucleotide Synthesis, CBT, Novum (Huddinge, Sweden) were used as sequencing primers. The sequencing reaction products were resolved on 8% polyacrylamide-urea gels. Gels were run at 40W for 1-3 h on a Sequencing Unit from Cambridge Electrophoresis Ltd (Cambridge, England), fixed in 10% methanol, 10% acetic acid for 15 min and dried on Whatman 3MM papers under vacuum. DNA bands were visualized by autoradiography. DNA fragments containing the fib gene from strain FDA 486 and strain Newman were produced by the polymerase chain reaction (PCR) using a Perkin Elmer Cetus DNA Thermal Cycler (Perkin Elmer, Norwalk, CT) and Taq polymerase (Boehringer-Mannheim). The PCR generated DNA fragments were sequenced in an Applied Biosystems 373A DNA Sequencer (Applied Biosystems Inc., Foster City, CA) using fluorescent nucleotide terminators (). Computer assisted analysis of DNA sequences was performed with GCG software package (Genetics Computer Group 1991) and with Seq Ed software (ref).

Competition in fibrinogen binding

The three different fibrinogen binding proteins from S. aureus 19, 60 and 87 kD are identified. We show that each of the FgBPs, separately or together with an additive effect can block S. aureus binding to immobilized fibrinogen in vitro. In short, the experimental procedure was as follows: Microtiter plates were coated with fibrinogen free of contaminating plasma components (IMCO). After-coating to block non specific adherence was done with BSA. Radiolabelled bactera were added, 2-5 x 106 per well. Simultaneously, various amounts of 19, 60 and/or 87 kD FgBP

were added. Bacterial adherence was measured after two hours incubation.

Figure 12: Binding of radiolabelled S. aureus to fibrinogen in the presence of various amounts of 19 kD FgBP.

Figure 13: Binding of radiolabelled S. aureus to fibrinogen in the presence of various amounts of 60 kD FgBP.

Figure 14: Binding of radiolabelled S. aureus to fibrinogen in the presence of various amounts of 87 kD FgBP.

Figure 15: Binding of radiolabelled S. aureus to fibrinogen in the presence of various combinations of FgBPs.

Incidence of FgBPs

The incidence of the 19 and the 87 kD FgBPs were measured. Thirty nine S. aureus isolates of human origin and thirty seven bovine mastitis isolates, taken from a wide variety of sources, were tested by PCR for the gene and in affinity blotting for the proteins.

All (100%) of the human isolates were positive in both PCR and affinity blotting for the 19 kD protein and 95% were positive for the 87 kD (only tested by affinity blotting).

Of the bovine isolates, 45% were positive in affinity blot for the 19 kD but 95% in PCR (some variation in the genome size was found). Fifty five were positive for the 87 kD FgBP.

Vaccination

The 19 and 87 kD proteins in combination were used to immunize mice which were subsequently subjected to experimental mastitis caused by S. aureus. A control group was given only the adjuvant (Freund's). Histopathological

examination and bacterial count was performed after 24 hours. A significant ($p<0.05$) difference in the number of colonizing bacteria was found between the two groups.

The present fibrinogen binding proteins can be used in immunization, whereby the proteins, preferably in combination with a fusion protein in order to form a larger antigen to react upon, are injected in doses creating an immunological reaction in the host mammal. Thus the fibrinogen binding proteins can be used in vaccination of mammals to protect against infections caused by staphylococcal infections.

Further, the fibrinogen binding proteins can be used to block an infection in an open skin lesion. Wounds can be treated by using a suspension comprising the fibrinogen binding protein. Thus the fibrinogen binding proteins can be used to treat wounds, e.g., for blocking bacterial binding sites in fibrinogen, or for immunization (vaccination). In the latter case the host produces specific antibodies which can protext against attachment by bacterial strains comprising such fibrinogen binding proteins. Hereby the antibodies block the adherence of the bacterial strains to damaged tissue.

Examples of colonizing of tissue damage are:

a) colonizing of wounds in skin and connective tissue, which wounds have been caused by a mechanical trauma, chemical damage, and/or thermical damage;

b) colonizing of wounds on mucous membranes such as in the mouth cavity, or in the mammary glands, urethra or vagina;

c) colonizing of connective tissue proteins, which have been exposed by minimal tissue damage (micro lesions) in connection with epithelium and endothelium (mastitis, heart valve infection, hip exchange surgery).

When using the present fibrinogen binding proteins, prepared by isolation from living cells, by means of

hybrid-DNA technique, or synthesized, for immunization (vaccination) in mammals, including humans, the proteins, or polypeptides thereof, are dispersed in sterile isotonic saline solution, optionally while adding a pharmaceutically acceptable dispersing agent. Different types of adjuvants can further be used in order to sustain the release in the tissue, and thus expose the protein for a longer period of time to the immuno defence system of a body.

A suitable dose to obtain immunization is 0.5 to 5 /µg of fibrinogen binding protein per kg body weight and injection at immunization. In order to obtain durable immunization, vaccinations should be carried out at consecutive occasions with an interval of 1 to 3 weeks, preferably at three occasions. Adjuvants are normally not added when repeating the immunization treatment.

When using the present fibrinogen binding proteins or polypeptides thereof for local topical administration the protein is dispersed in an isotonic saline solution to a concentration of 25 to 250 /µg per ml. The wounds are then treated with such an amount only to obtain a complete wetting of the wound surface. For an average wound thus only a couple of millilitres of solution are used in this way. After treatment using the protein solution the wounds are suitably washed with isotonic saline solution or another suitable wound treatment solution.

Further the fibrinogen binding protein, or synthetized polypeptide thereof can be used to diagnoze bacterial infections caused by Staphylococcus aureus strains, whereby a fibrinogen binding protein of the present invention is immobilized on a solid carrier, such as small latex or Sepharose^{R} beads, whereupon sera containing antibodies are allowed to pass and react with the fibrinogen binding protein thus immobilized. The agglutination is then measured by known methods.

Further the fibrinogen binding protein or polypeptide can be used in an ELISA test (Enzyme Linked Immuno Sorbent Assay; E Engvall, Med. Biol. 55, 193 (1977)). Hereby wells in a polystyrene microtitre plate are coated with the fibrinogen binding protein and incubated over night at 4°C. The plates are then thoroughly washed using PBS containing 0.05% Tween 20, and dried. Serial dilutions of the patient serum made in PBS-Tween, are added to the wells, and are incubated at 30°C for 1.5 hrs. After rinsing anti-human IgG conjugated with an enzyme, or a horseradish peroxidase, or an alkaline phosphatase is added to the wells and further incubated at 30°C for 1.5 hrs. During these incubations IgG from patient serum, and added anti-human IgG-enzyme conjugate, respectively, has been bound thereto. After rinsing, an enzyme substrate is added, p-nitrophosphate in case of an alkaline phosphatase, or orthophenylene diamine substrate (OPD) in case a peroxidase has been used, respectively. The wells of the plates are then rinsed using a citrate buffer containing 0.055% OPD, and 0.005% H_2O_2, and incubated at 30°C for 10 min. The enzyme reaction is stopped by adding a 4N solution of H_2SO_4 to each well. The colour development is measured using a spectrophotometer.

Depending on the type of enzyme substrate used a fluoroscence measurement can be used as well.

Another method to diagnoze S. aureus infections is by using the DNA gene probe method based on the nucleotide sequence for the fibrinogen binding protein or part thereof. Thereby the natural or synthetic DNA sequence is attached to a solid carrier, such as a nitrocellulose filter, a nylon filter, or a polystyrene plate as mentioned above, by e.g., adding a body fluid, to the surface. The DNA gene probe, optionally labelled enzymatically, or by a radioactive isotope, is then added to the solid surface plate comprising the DNA sequence, whereby the DNA gene

probe attaches to the membrane associated sequence where appearing. The enzyme or radioactive isotope can readily be determined by known methods.

Above the term fibrinogen binding protein includes any of polypeptide thereof as well, which constitute the minimal fibrinogen binding site of the complete protein.

The fibrinogen binding protein/s can be used for raising antibodies by administering the protein and then isolating said antibodies, whereupon these are administered for passive immunization purposes.

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Figure legends

Fig. 1. Analysis of affinity purified material from fibrinogen-Sepharose. Arrows indicate molecular masses (in kDa). Immunoblot probed with anti-19 serum. Lanes: 1, fibrinogen-proteins from S. aureus strain Newman; 2, fibrinogen-proteins from S. aureus strain FDA 486; 3, fibrinogen-proteins from E. coli XL-1 harbouring plasmid pBfibIII; 4, fibrinogen-proteins from E. coli XL-1 harbouring plasmid pBfibT.

Fig. 2. Restriction map and sequencing strategy of the insert containing the fib gene. Subcloning of the fib gene from the original λ clone on a HindIII - HindIII fragment resulted in the pBfibIII vector. This was further subcloned into the pBfibT and pBfib J vectors. Boxes show the regions for which the sequence was deduced. SS denotes the signal sequence and fib the structural gene for the mature fib protein. Arrows indicate the primers used for sequencing.

Fig. 3. Nucleotide and amino acid sequence for the fib protein gene. The box denotes a possible Shine-Dalgarno sequence. Putative promoter sequences are underlined. The vertical arrow indicates the cleavage site of the signal sequence.

Fig. 4. Comparison of the nucleotide sequences for the fib gene from strain FDA 486 (top sequence) and strain Newman. Similarity is shown by blank spaces, differences in sequence is indicated by the diverging nucleotide of the Newman fib gene.

Fig. 5. Comparison of the amino acid sequences for the fib protein from strain FDA 486 (top sequence) and strain Newman. Similarity is shown by blank spaces, differences in sequence is indicated by the diverging amino acid of the Newman protein.

Fig. 6. Sequence homologies between the fib protein and the coagulase from S. aureus. Bold letter show

homologies between the two repeats in the fib protein. Shaded letters show homologies between the fib protein and coagulase.

Fig. 7. Coomassie blue-stained SDS-PAGE of fibrinogen-binding material, affinity purified from S. aureus culture supernatants. Cells were grown in LB under low aeration conditions and samples were taken every hour. Lanes 1-6 represent samples taken after 1, 2, 3, 5, 7 and 9 h.

Fig. 8. Analysis of affinity purified material from fibrinogen- and prothrombin-Sepharose. (a) Coomassie blue stained, undiluted eluate; (b) Immunoblot of eluate (diluted 1/100), probed with fibrinogen (10 µg/ml) and pre-absorbed antifibrinogen antibody; (c) immunoblot of eluate (diluted 1/100), probed with prothrombin (10 µg/ml) and pre-absorbed antiprothrombin antibody. Lanes: 1, eluate from fibrinogen-Sepharose purified from culture supernatants of staphylococci grown in BHI for 3-4 h; 2, eluate from prothrombin-Sepharose purified from culture supernatants of staphylococci grown in LB for 6-8 h and initially passed through fibrinogen-Sepharose.

Fig. 9. Immunoblot analysis of eluate from fibrinogen-Sepharose. Lanes: 1, eluate (undiluted) incubated with fibrinogen (20 ng/ml) and antifibrinogen antibody; 2, eluate (undiluted) incubated with anti-19 serum.

Fig. 10. Immunoblot analysis of eluate (diluted 1/100) from fibrinogen- and prothrombin-Sepharose prepared as indicated in Fig. 2. (a) Anti-19 serum pre-absorbed with the 60-kDa protein; (b) Anti-19 serum pre-absorbed with the 19-kDa protein. Lanes: 1, eluate from fibrinogen-Sepharose; 2, eluate from prothrombin-Sepharose.

Fig. 11. Analysis of purified proteins eluted from preparative SDS-PAGE gels. (a) Silver stain of undigested sample; (b-d) immunoblots probed with fibrinogen and antifibrinogen antibodies; (b) undigested sample; (c)

samples digested with α-chymotrypsin; (d) samples digested with staphylococcal V8 protease. Lanes: 1, 19 kDa protein; 2, 87 kDa protein; 3, 60 kDa protein.

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ACGATGGTAC ATTCGAATAT GGTGCACGTC CACAATTTAA TAAACCAGCA
GCGAAAACTG ATGCAACTAT TAAAAAAGAA CAAAAATTGA TTCAAGCTCA
AAATCTTGTG AGAGAATTTG AAAAAACACA TACTGTCAGT GCACACAGAA
AAGCACAAAA GGCAGTCAAC TTAGTTTCGT TTGAATACAA AGTGAAGAAA
ATGGTCTTAC AAGAGCGAAT TGATAATGTA TTAAAACAAG GATTAGTGAG
ATAATACTTC TGTCATTATT TTAAGTTCAA AATAATTTAA TATTATATTA
TTTTTTATTA ATAAAACGAC TATGCTATTT AATGCCAGGT TAATGTAACT
TTCCTAAAAT TGACTATATA ATCGTTAAGT ATCAATTTTA AGGAGAGTTT
ACAATGAAAT TTAAAAAATA TATATTAACA GGAACATTAG CATTACTTTT
ATCATCAACT GGGATAGCAA CTATAGAAGG GAATAAAGCA GATGCAAGTA
GTCTGGACAA ATATTTAACT GAAAGTCAGT TTCATGATAA ACGCATAGCA
GAAGAATTAA GAACTTTACT TAACAAATCG AATGTATATG CATTAGCTGC
AGGAAGCTT

ATAGATAACT ATATTTTGTC TATATTATAA AGTGTTTATA GTTAATTAAT
AATTAGTTAA TTTCAAAAGT TGTATAAATA GGATAACTTA ATAAATGTAA
GATAATAATT TGGAGGATAA TTAACATGAA AAATAAATTG ATAGCAAAAT
CTTTATTAAC AATAGCGGCA ATTGGTATTA CTACAACTAC AATTGCGTCA
ACAGCAGATG CGAGCGAAGG ATACGGTCCA AGAGAAAAGA AACCAGTGAG
TATTAATCAC AATATCGTAG AGTACAATGA TGGTACTTTT AAATATCAAT
CTAGACCAAA ATTTAACTCA ACACCTAAAT ATATTAAATT CAAACATGAC
TATAATATTT TAGAATTTAA CGATGGTACA TTCGAATATG GTGCACGTCC
ACAATTTAAT AAACCAGCAG CGAAAACTGA TGCAACTATT AAAAAAGAAC
AAAAATTGAT TCAAGCTCAA AATCTTGTGA GAGAATTTGA AAAAACACAT
ACTGTCAGTG CACACAGAAA AGCACAAAAG GCAGTCAACT TAGTTTCGTT
TGAATACAAA GTGAAGAAAA TGGTCTTACA AGAGCGAATT GATAATGTAT
TAAAACAAGG ATTAGTGAGA TAATACTTCT GTCATTATTT TAAGTTCAAA
A....TAATT TAATATTATA TTATTTTTTA TTAATAAAAC GACTATGCTA
TTTAATGCCA GGTTAATGTA ACTTTCCTAA AATTGACTAT ATAATCGTTA
AGTATCAATT TTAAGGAGAG TTTACAATGA AATTT

MKNKLIAKSL LTIAAIGITT TTIASTADAS EGYGPREKKP VSINHNIVEY
NDGTFKYQSR PKFNSTPKYI KFKHDYNILE FNDGTFEYGA RPQFNKPAAK
TDATIKKEQK LIQAQNLVRE FEKTHTVSAH RKAQKAVNLV SFEYKVKKMV
LQERIDNVLK QGLVR

CLAIMS

1. A new fibrinogen binding protein derived from Staphylococci having a molecular weight of 60 kDa.

2. A new fibrinogen binding protein derived from Staphylococci having a molecular weight of 19 kDa.

3. A fibrinogen binding protein according to claim 2, having the NH_2-terminal sequence SEGYG PREKK PVSIN HNIVE.

4. A fibrinogen binding protein of claim 1 having coagulase activity.

5. Hybrid-DNA-molecule comprising a nucleotide sequence from S. aureus coding for a protein or polypeptide having fibrinogen binding activity.

6. Plasmid or phage comprising a nucleotide sequence from S. aureus coding for a protein or polypeptide having fibrinogen binding activity.

7. A plasmid pBfibIII as contained in E. coli XL having the deposit number DSM .....

8. An E. coli strain expressing said fibrinogen binding protein.

9. A microorganism transformed by recombinant DNA molecule of claims 5-7.

10. Hybrid-DNA-molecule according to claim 5, characterized in that it comprises the following nucleotide sequence:

GAGCGAAGGA TACGGTCCAA GAGAAAAGAA ACCAGTGAGT ATTAATCACA
ATATCGTAGA GTACAATGAT GGTACTTTTA AATATCAATC TAGACCAAAA
TTTAACTCAA CACCTAAATA TATTAAATTC AAACATGACT ATAATATTTT
AGAATTTAAC GATGGTACAT TCGAATATGG TGCACGTCCA CAATTTAATA
AACCAGCAGC GAAAACTGAT GCAACTATTA AAAAAGAACA AAAATTGATT
CAAGCTCAAA ATCTTGTGAG AGAATTTGAA AAAACACATA CTGTCAGTGC
ACACAGAAAA GCACAAAAGG CAGTCAACTT AGTTTCGTTT GAATACAAAG
TGAAGAAAAT GGTCTTACAA GAGCGAATTG ATAATGTATT AAAACAAGGA
TTAGTGAG

11. A method for producing a fibrinogen binding protein or polypeptide wherein a) at least one hybrid-DNA-molecule according to claim 5, is introduced into a microorganism, b) said microorganism is cultivated in a growth promoting medium, and c) the protein thus formed is isolated.

12. A fibrinogen binding protein or polypeptide comprising at least one amino acid sequence
SEGYGPREKK PVSINHNIVE YNDGTFKYQS RPKFNSTPKY IKFKHDYNIL EFNDGTFEYG ARPQFNKPAA KTDATIKKEQ KLIQAQNLVR EFEKTHTVSA HRKAQKAVNL VSFEYKVKKM VLQERIDNVL KQGLVR

13. Pharmaceutical composition for the inhibition of Staphylococci binding to fibrinogen comprising a fibrinogen binding protein of a preceding claim in combination with a pharmaceutically inert carrier.

14. Method for inhibition of Staphylococci binding to fibrinogen in mammals including humans, by administering a therapeutically and/or prophylactically effective amount of a fibrinogen binding protein of any preceding claim together with an inert carrier.

15. Method for immunization, characterized in that the protein is administered in an amount sufficient to raise antibodies.

16. Method for immunization, whereby an antibody raised in accordance with claim 15 is administered to a mammal body in amount sufficient to provide a passive immunization.

1 2 3 4

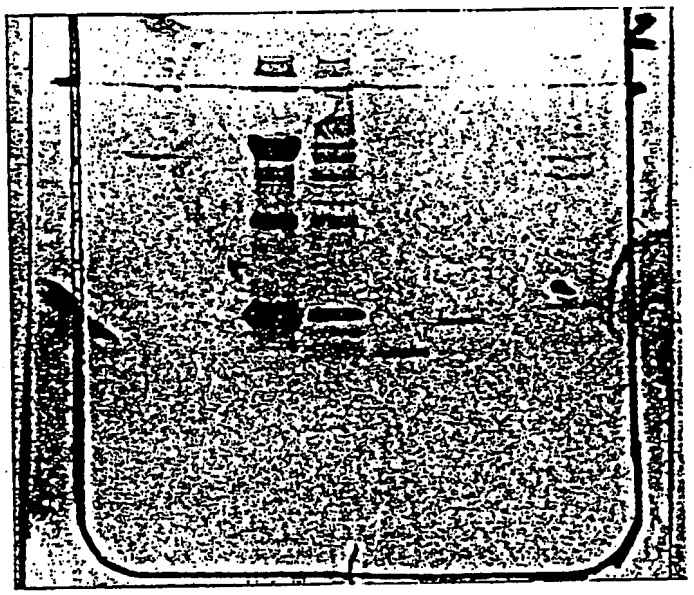

FIG 1

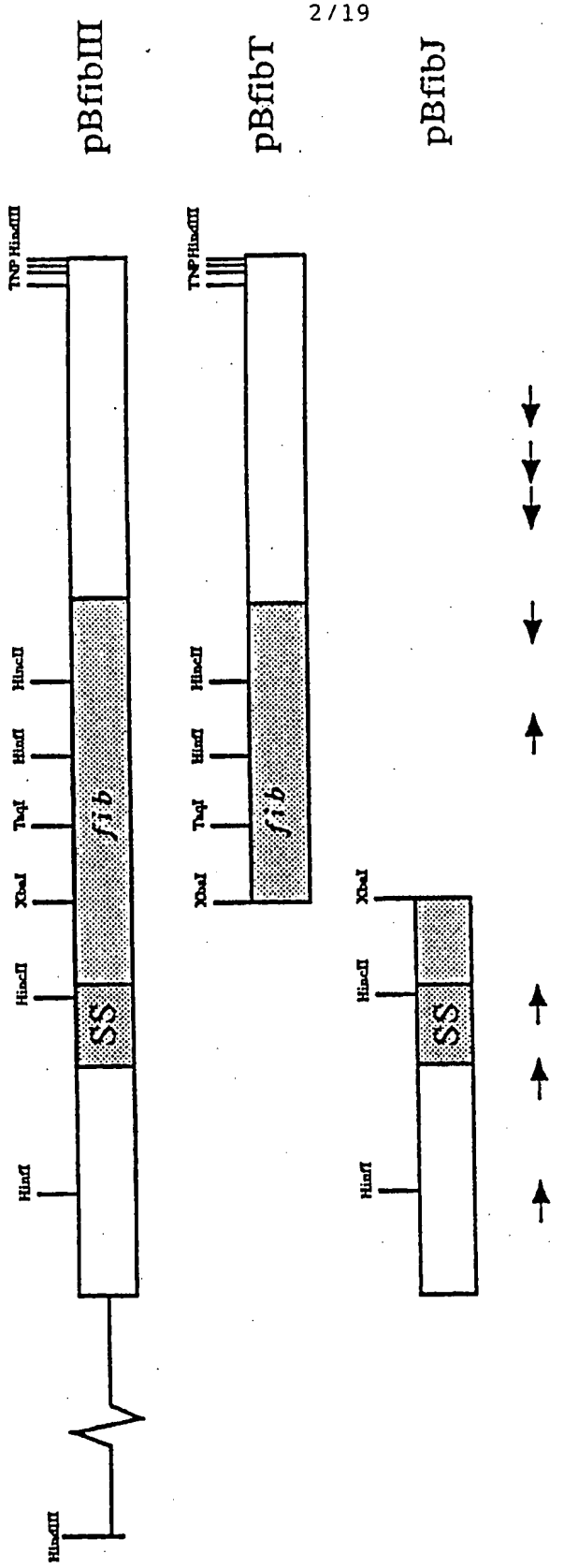
pBfibIII
pBfibT
pBfibJ
TNP HindIII
HincII
HindI
TaqI
XbaI
HincII
HindI
HindIII
fib
SS

FIG 2

```
1    GACTAGTGTATAAGTGCTGATGAGTCACAAGATAGATAACTATATTTTGTCTATATTATA    60
                                                      -35

61   AAGTGTTTATAGTTAATTAATAATTAGTTAATTTCAAAAGTTGTATAAATAGGATAACTT   120
                          -18                  -35

121  AATAAATGTAAGATAATAATTTGGAGGATAATTAACATGAAAAATAAATTGATAGCAAAA   180
       -18                                  M  K  N  K  L  I  A  K

181  TCTTTATTAACAATAGCGGCAATTGGTATTACTACAACTACAATTGCGTCAACAGCAGAT   240
     S  L  L  T  I  A  A  I  G  I  T  T  T  T  I  A  S  T  A  D

241  GCGAGCGAAGGATACGGTCCAAGAGAAAAGAAACCAGTGAGTATTAATCACAATATCGTA   300
     A  S  E  G  Y  G  P  R  E  K  K  P  V  S  I  N  H  N  I  V

301  GAGTACAATGATGGTACTTTTAAATATCAATCTAGACCAAAATTTAACTCAACACCTAAA   360
     E  Y  N  D  G  T  F  K  Y  Q  S  R  P  K  F  N  S  T  P  K

361  TATATTAAATTCAAACATGACTATAATATTTTAGAATTTAACGATGGTACATTCGAATAT.  420
     Y  I  K  F  K  H  D  Y  N  I  L  E  F  N  D  G  T  F  E  Y

421  GGTGCACGTCCACAATTTAATAAACCAGCAGCGAAAACTGATGCAACTATTAAAAAAGAA   480
     G  A  R  P  Q  F  N  K  P  A  A  K  T  D  A  T  I  K  K  E

481  CAAAAATTGATTCAAGCTCAAAATCTTGTGAGAGAATTTGAAAAAACACATACTGTCAGT   540
     Q  K  L  I  Q  A  Q  N  L  V  R  E  F  E  K  T  H  T  V  S

541  GCACACAGAAAAGCACAAAAGGCAGTCAACTTAGTTTCGTTTGAATACAAAGTGAAGAAA   600
     A  H  R  K  A  Q  K  A  V  N  L  V  S  F  E  Y  K  V  K  K

601  ATGGTCTTACAAGAGCGAATTGATAATGTATTAAAACAAGGATTAGTGAGATAATACTTC   660
     M  V  L  Q  E  R  I  D  N  V  L  K  Q  G  L  V  R  *

661  TGTCATTATTTTAAGTTCAAAATAATTTAATATTATATTATTTTTTATTAATAAAACGAC   720

721  TATGCTATTTAATGCCAGGTTAATGTAACTTTCCTAAAATTGACTATATAATCGTTAAGT   780

781  ATCAATTTTAAGGAGAGTTTACAATGAAATTTAAAAAATATATATTAACAGGAACATTAG   840
                                  M  K  F  K  K  Y  I  L  T  G  T  L  A

841  CATTACTTTTATCATCAACTGGGATAGCAACTATAGAAGGGAATAAAGCAGATGCAAGTA   900
       L  L  L  S  S  T  G  I  A  T  I  E  G  N  K  A  D  A  S  S

901  GTCTGGACAAATATTTAACTGAAAGTCAGTTTCATGATAAACGCATAGCAGAAGAATTAA   960
       L  D  K  Y  L  T  E  S  Q  F  H  D  K  R  I  A  E  E  L  R

961  GAACTTTACTTAACAAATCGAATGTATATGCATTAGCTGCAGGAAGCTT  1009
       T  L  L  N  K  S  N  V  Y  A  L  A  A  G  S    1
```

FIG 3

```
1   ATAGATAACTATATTTTGTCTATATTATAAAGTGTTTATAGTTAATTAATAATTAGTTAA   60
1          G        CA                                             60

61  TTTCAAAAGTTGTATAAATAGGATAACTTAATAAATGTAAGATAATAATTTGGAGGATAA  120
61                                                                 120

121 TTAACATGAAAAATAAATTGATAGCAAAATCTTTATTAACAATAGCGGCAATTGGTATTA  180
121   G            GC                          T          A        180

181 CTACAACTACAATTGCGTCAACAGCAGATGCGAGCGAAGGATACGGTCCAAGAGAAAAGA  240
181                                                                240

241 AACCAGTGAGTATTAATCACAATATCGTAGAGTACAATGATGGTACTTTTAAATATCAAT  300
241                                                                300

301 CTAGACCAAAATTTAACTCAACACCTAAATATATTAAATTCAAACATGACTATAATATTT  360
301                                                                360

361 TAGAATTTAACGATGGTACATTCGAATATGGTGCACGTCCACAATTTAATAAACCAGCAG  420
361                                                                420

421 CGAAAACTGATGCAACTATTAAAAAAGAACAAAAATTGATTCAAGCTCAAAATCTTGTGA  480
421                                                                480

481 GAGAATTTGAAAAAACACATACTGTCAGTGCACACAGAAAAGCACAAAAGGCAGTCAACT  540
481                                                                540

541 TAGTTTCGTTTGAATACAAAGTGAAGAAAATGGTCTTACAAGAGCGAATTGATAATGTAT  600
541                                                                600

601 TAAAACAAGGATTAGTGAGATAATACTTCTGTCATTATTTTAAGTTCAAAA....TAATT  660
601                  T A    A     AA  G  GC G T TC GG   TAAT        660

661 TAATATTATATTATTTTTTATTAATAAAACGACTATGCTATTTAATGCCAGGTTAATGTA  720
661 A   G    G   A     G     G   AA G  AT          A               720

721 ACTTTCCTAAAATTGACTATATAATCGTTAAGTATCAATTTTAAGGAGAGTTTACAATGA  780
721  T G    G              C  AG                        C   T     780

781 AATTT  785
781        785
```

FIG 4

```
1   MKNKLIAKSLLTIAAIGITTTTIASTADASEGYGPREKKPVSINHNIVEYNDGTFKYQSR   60
1     A       L                                                    60

61  PKFNSTPKYIKFKHDYNILEFNDGTFEYGARPQFNKPAAKTDATIKKEQKLIQAQNLVRE  120
61                                                                 120

121 FEKTHTVSAHRKAQKAVNLVSFEYKVKKMVLQERIDNVLKQGLVR  165
121                                                K  165
```

FIG 5

Fbg-bp, strain Newman:

SEGYGPR

EKKPVSINH **NIVEYNDGSFK YQSRPKFNSTP**

KYIKFKHDY **NILEFNDGTFE YGARPQFNKPA**

AKTDATIKKEQKLIQAQNLVREFEKTHTVSAHRKAQKAVNLVSFEYKVKKMVLQERIDNVLKQGLVR

Coagulase, strain 8325-4:

(C-terminal fragment)

ASQ **YGPRPQFNKTP**

KYVKYRDAGT **GIREYNDGTFG YEARPRFNKPS**

ETNAY **NVTTHANGQVS YGARPTYKKPS**

ETNAY **NVTTHANGQVS YGARPTQNKPS**

KTNAY **NVTTHGNGQVS YGARQAQNKPS**

KTNAY **NVTTHANGQVS YGARPTYKKPS**

KTNAY **NVTTHADGTAT YGPRVTK**

FIG 6

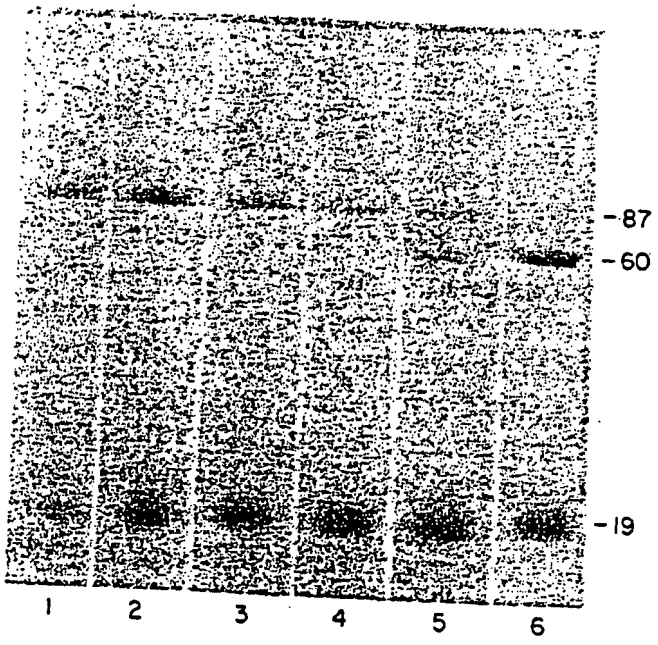

FIG 7

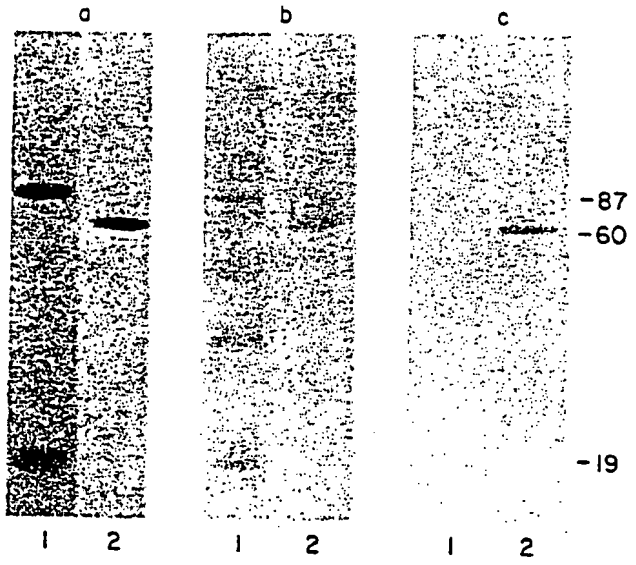

FIG 8

FIG 9

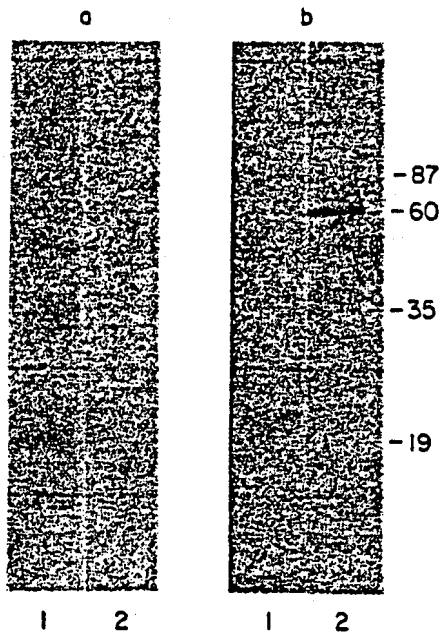

FIG 10

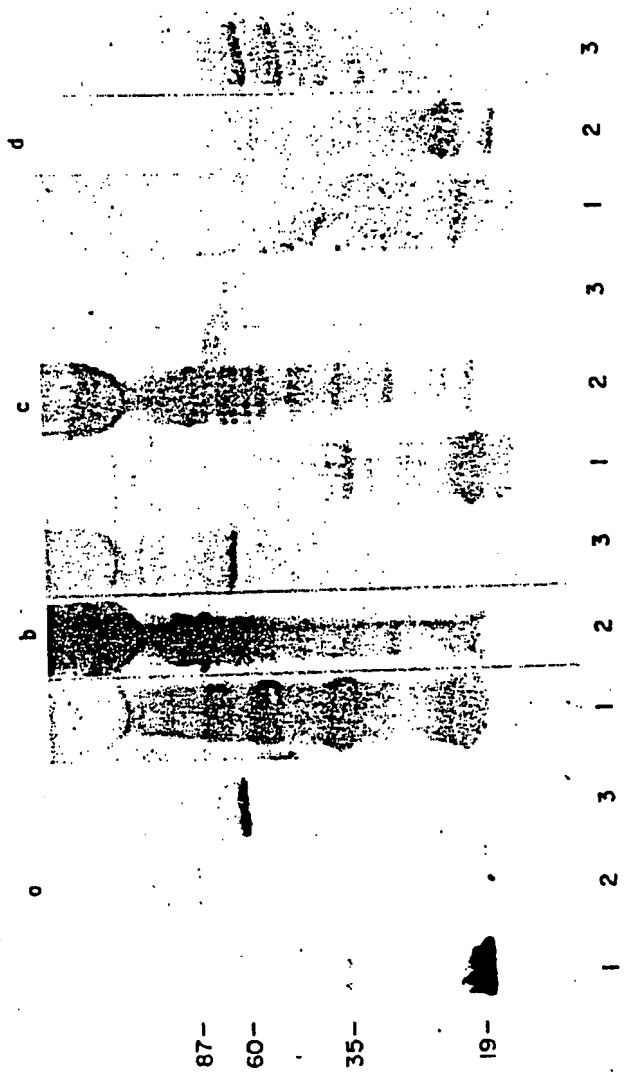

FIG 11

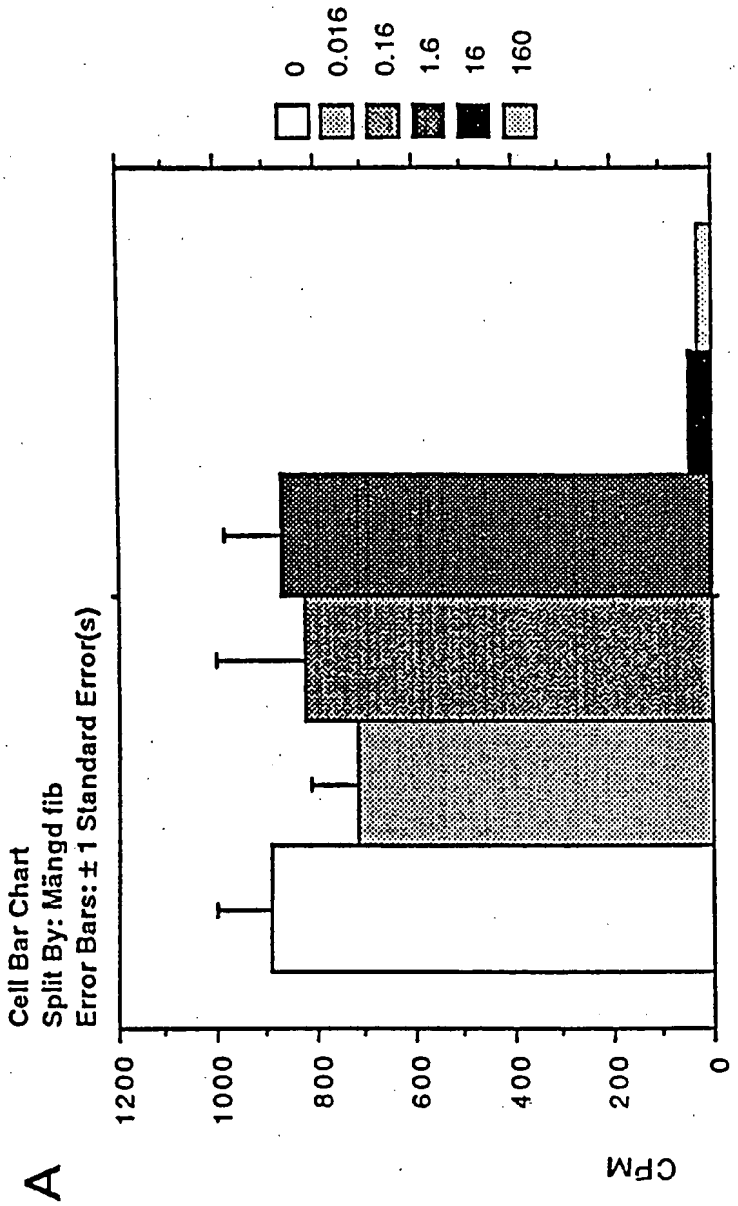
A
Cell Bar Chart
Split By: Mängd fib
Error Bars: ± 1 Standard Error(s)
0
0.016
0.16
1.6
16
160
CPM
0
200
400
600
800
1000
1200

FIG 12

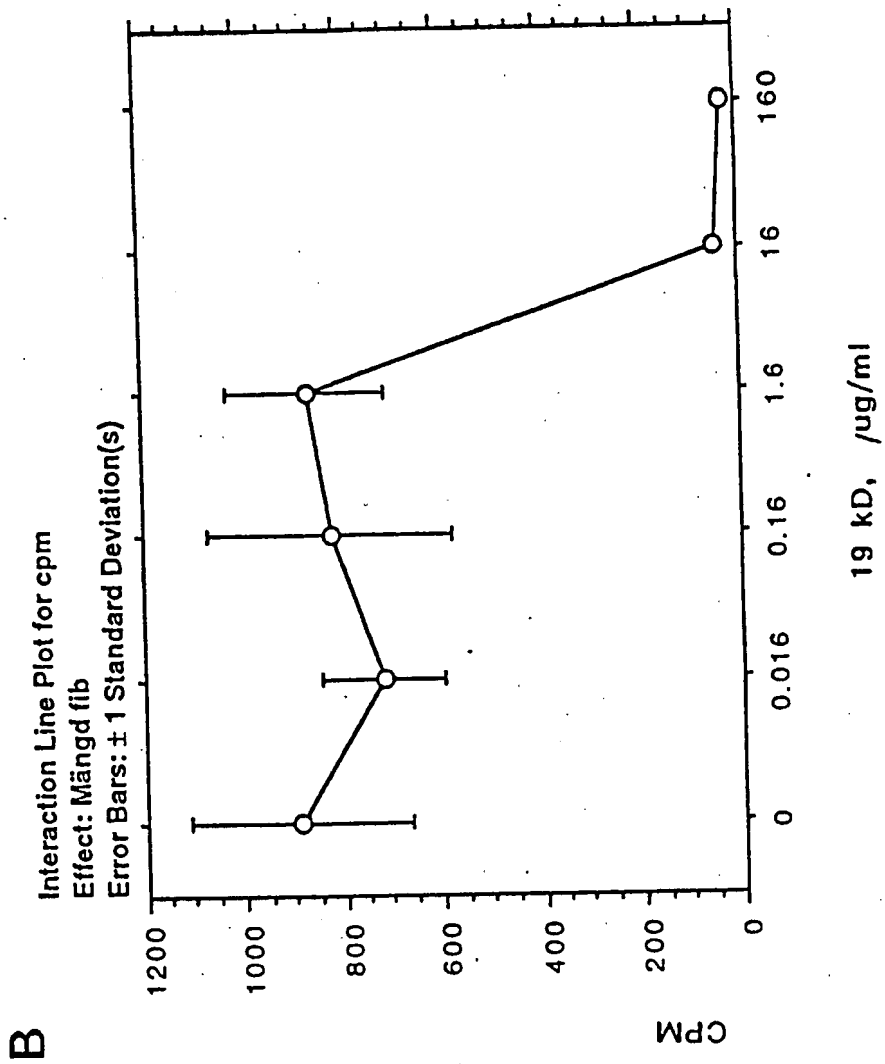
B
Interaction Line Plot for cpm
Effect: Mängd fib
Error Bars: ± 1 Standard Deviation(s)
CPM
1200
1000
800
600
400
200
0
0
0.016
0.16
1.6
16
160
19 kD, /ug/ml

FIG 12

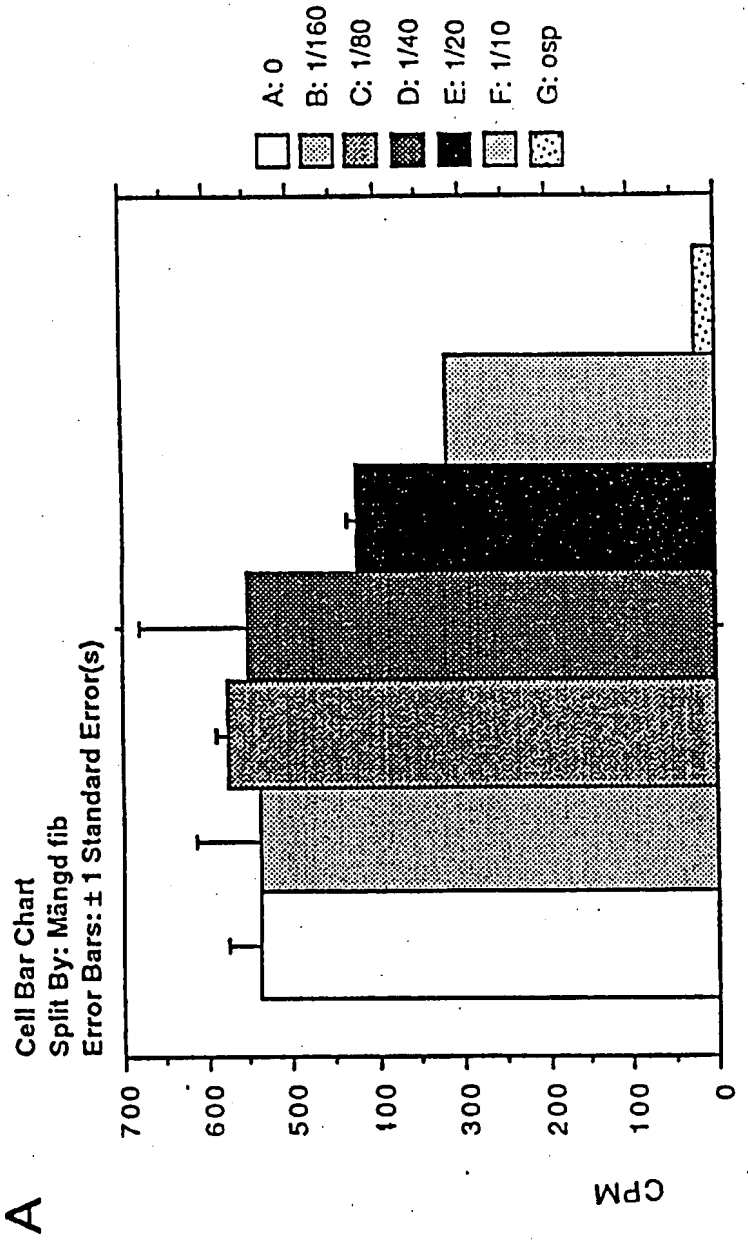
A
Cell Bar Chart
Split By: Mängd fib
Error Bars: ± 1 Standard Error(s)
A: 0
B: 1/160
C: 1/80
D: 1/40
E: 1/20
F: 1/10
G: osp
CPM
700
600
500
100
300
200
100
0

FIG 13

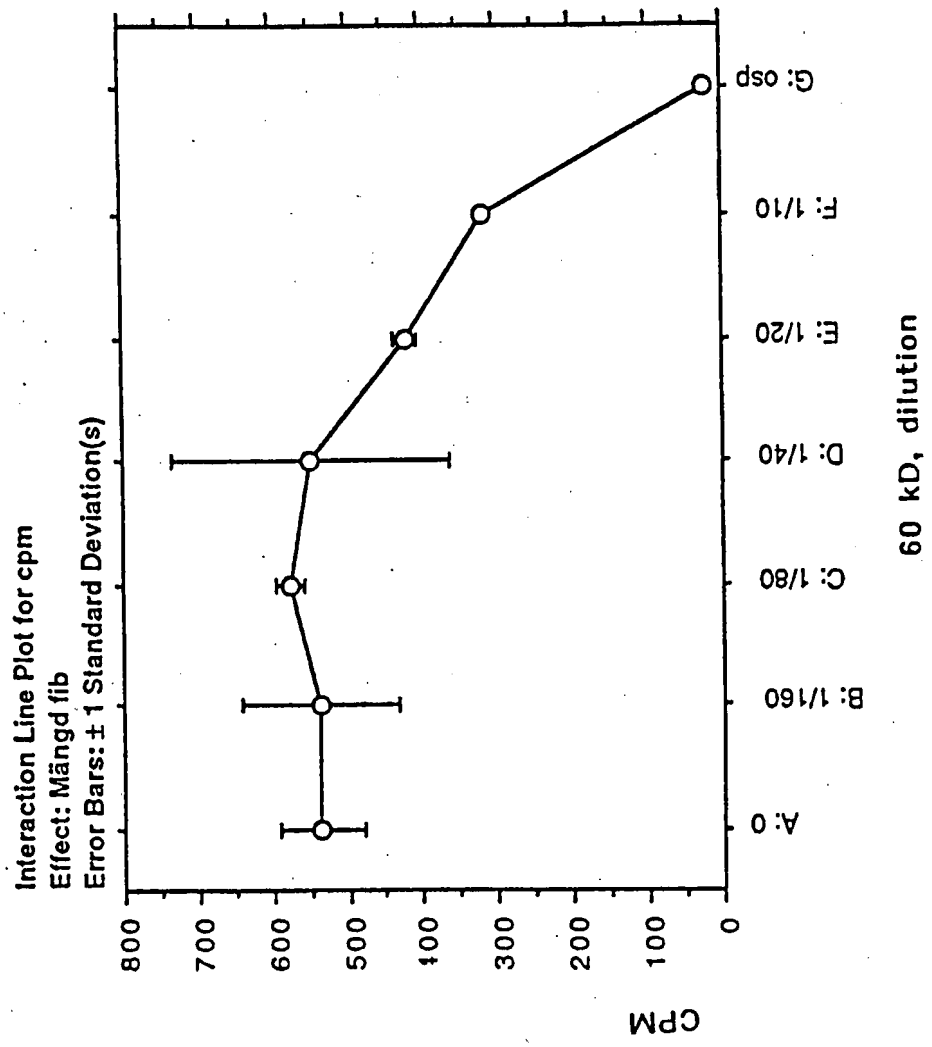
B
Interaction Line Plot for cpm
Effect: Mängd fib
Error Bars: ± 1 Standard Deviation(s)
CPM
800
700
600
500
100
300
200
100
0
A: 0
B: 1/160
C: 1/80
D: 1/40
E: 1/20
F: 1/10
G: osp
60 kD, dilution

FIG 13

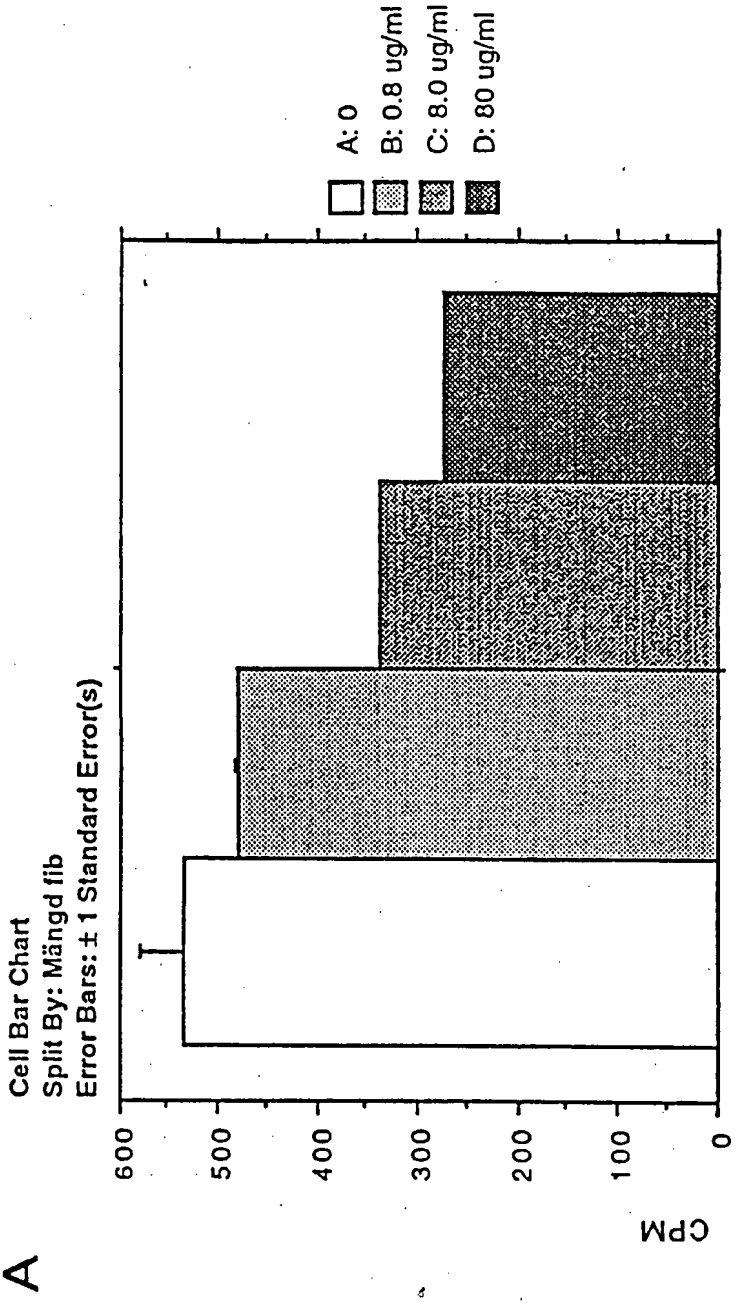
A: 0
B: 0.8 ug/ml
C: 8.0 ug/ml
D: 80 ug/ml
Cell Bar Chart
Split By: Mängd fib
Error Bars: ± 1 Standard Error(s)
600
500
400
300
200
100
0
CPM
A

FIG 14

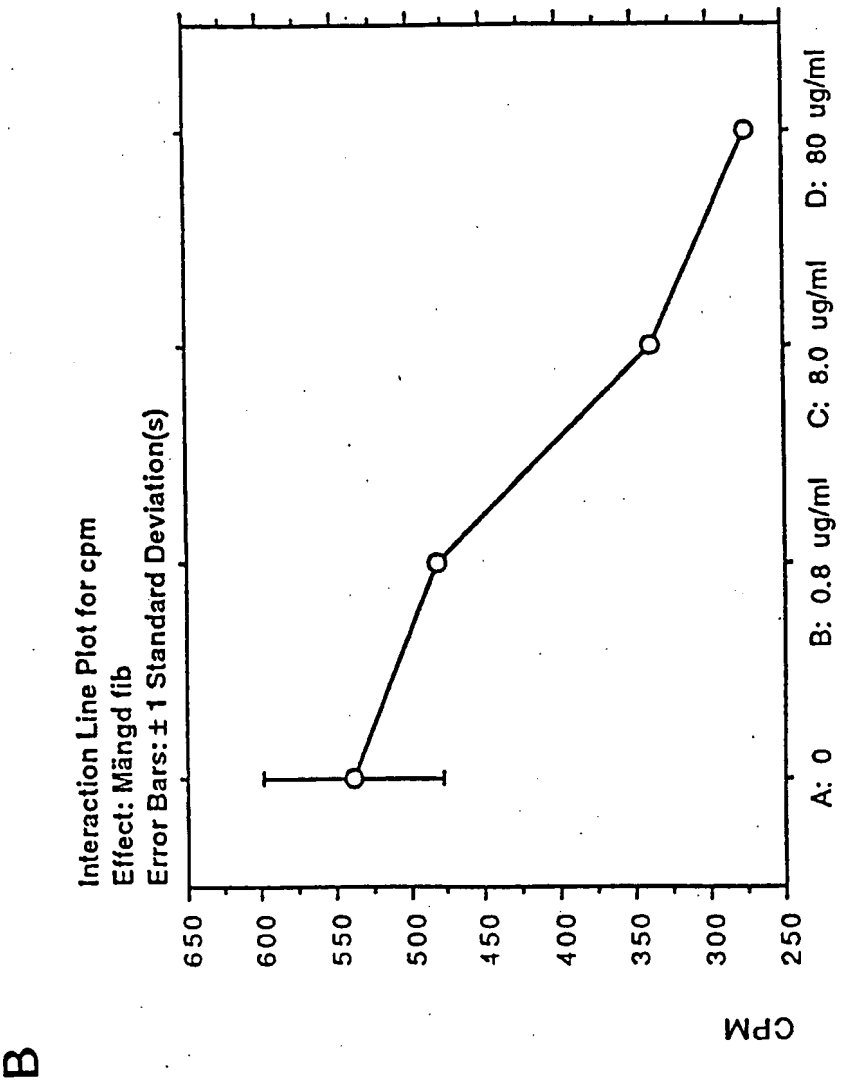
B
Interaction Line Plot for cpm
Effect: Mängd fib
Error Bars: ± 1 Standard Deviation(s)
650
600
550
500
450
400
350
300
250
CPM
A: 0
B: 0.8 ug/ml
C: 8.0 ug/ml
D: 80 ug/ml
87 kD, /ug/ml

FIG 14

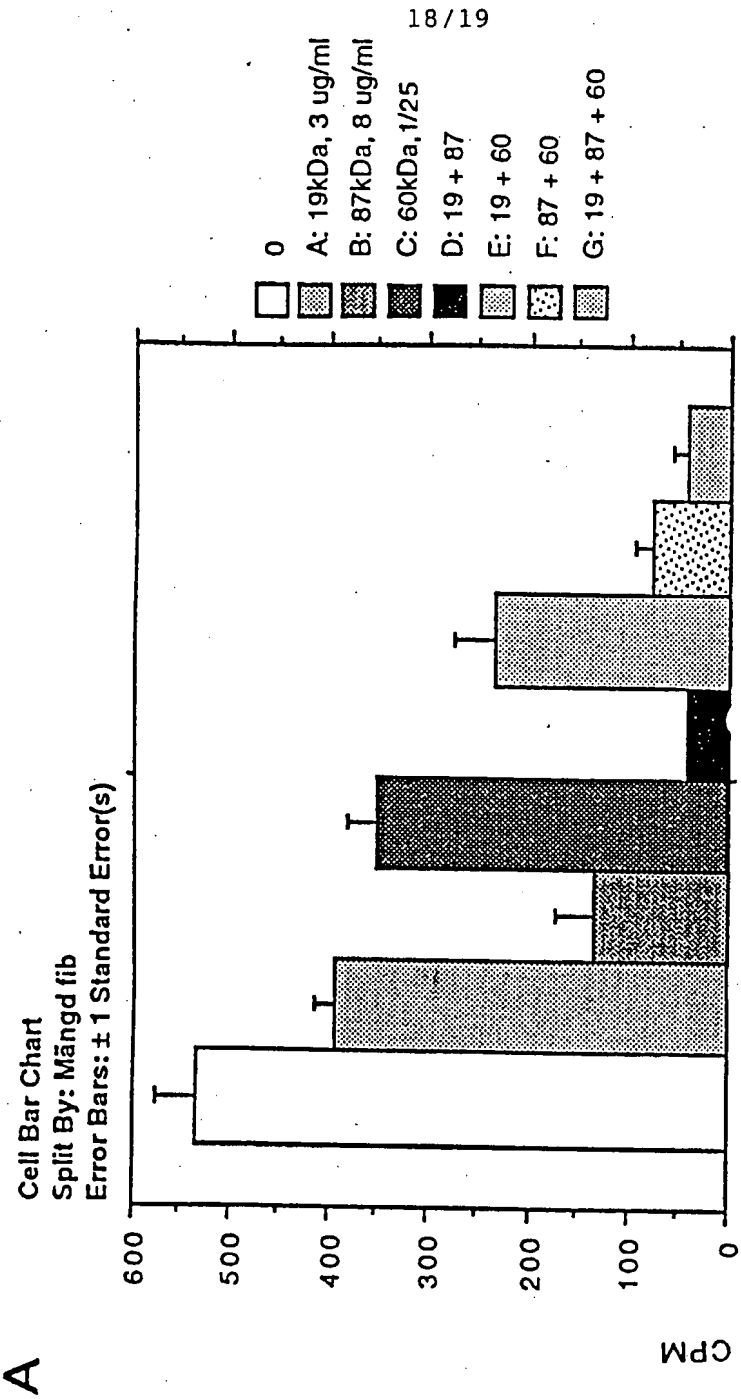
A
Cell Bar Chart
Split By: Mängd fib
Error Bars: ± 1 Standard Error(s)
CPM
600
500
400
300
200
100
0
0
A: 19kDa, 3 ug/ml
B: 87kDa, 8 ug/ml
C: 60kDa,1/25
D: 19 + 87
E: 19 + 60
F: 87 + 60
G: 19 + 87 + 60

FIG 15

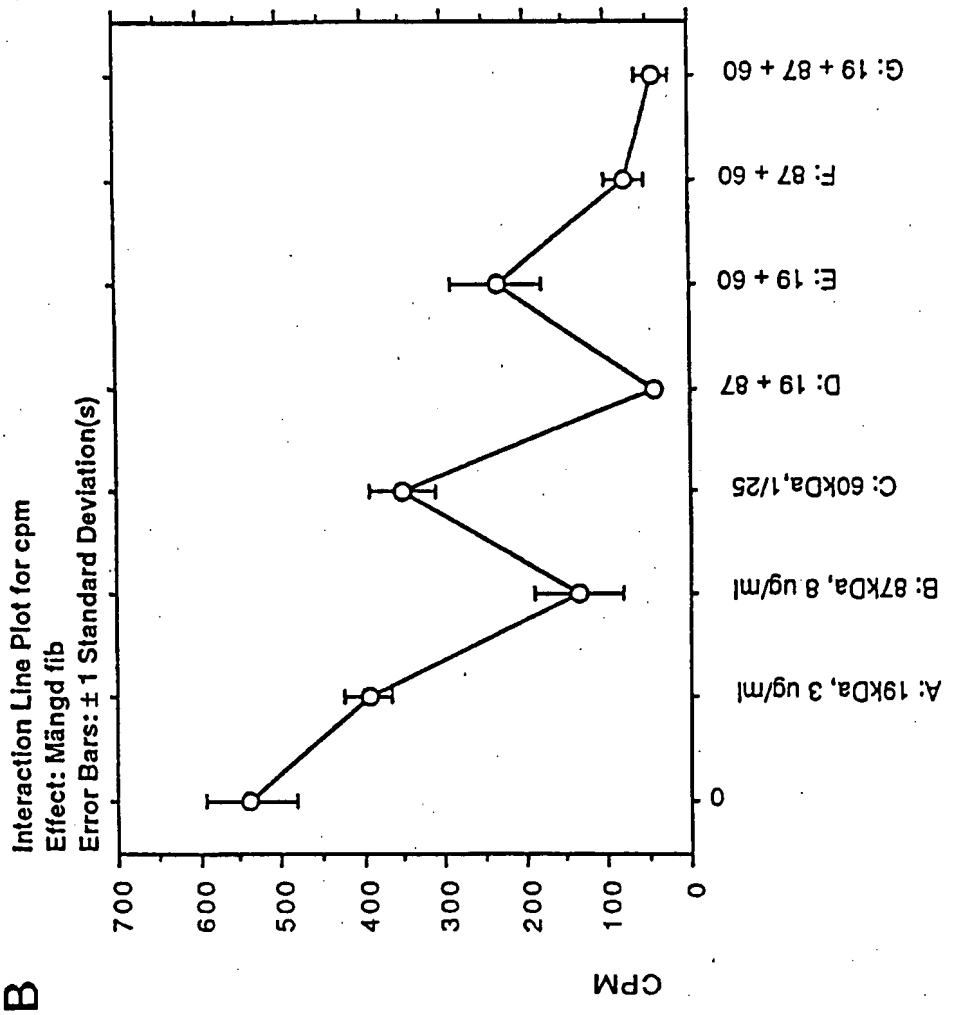
B
Interaction Line Plot for cpm
Effect: Mängd fib
Error Bars: ± 1 Standard Deviation(s)
CPM
700
600
500
400
300
200
100
0
0
A: 19kDa, 3 ug/ml
B: 87kDa, 8 ug/ml
C: 60kDa,1/25
D: 19 + 87
E: 19 + 60
F: 87 + 60
G: 19 + 87 + 60
Inhibition using 19, 87, and 60 kD

FIG 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00759

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 15/04, C12N 9/48, C12N 15/31, A61K 39/085, A61K 37/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EMBL, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL Protein sequence database, Accession number S34269, May 1993, M.K. Boden et al.	1-13
X	Dialog Information Services, File 154, Medline, Dialog accession no. 08196128, Boden M.K. et al: "Evidence for three different fibrinogen binding proteins with unique properties from Staphylococcus aureus strain Newman", & Microb Pathoq Apr 1992, 12 (4) p289-98	1-13
X	Dialog Information Services, file 154, Medline, Dialog accession no: 07005549, Boden M.K. et al: "Fibrinogen binding protein/clumping factor from Staphylococcus aureus", & Infect Immun Aug 1989, 57 (8) p2358-63	2,5-9,11,13

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 1994	11 -01- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Mikael G:son Bergstrand Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00759

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 154, Medline, Dialog accession no: 07005549, Boden M.K. et al: "Fibrinogen binding protein/clumping factor from Staphylococcus aureus", & Infect Immun Aug 1989, 57 (8) p2358-63 --	2,5-9,11,13
A	EP, A2, 0163623 (ALFA-LAVAL AGRI INTERNATIONAL AB), 4 December 1985 (04.12.85) --	1-13

INTERNATIONAL SEARCH REPORT

ational application No.

PCT/SE 93/00759

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14-16
because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body, c.f. PCT rule 39.1(iv).

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

27/11/93

International application No.
PCT/SE 93/00759

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0163623	04/12/85	SE-T3- 0163623	
		AU-B- 600886	30/08/90
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